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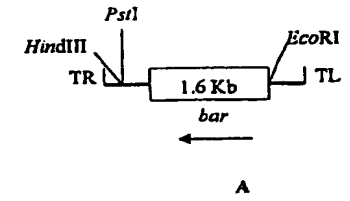
(54) Title: PLANT TRANSFORMATION PROCESS

(57) Abstract

A method of *Agrobacterium*–mediated genetic transformation using plants at the time of flowering has been found which is applicable to dicots and monocots capable of being transformed by *Agrobacterium*. The transformation method utilizes vacuum–infiltration to introduce the *Agrobacterium* T–DNA carrying a gene of interest into the plants, preferably plants grown from vernalized seed. Upon maturity, seeds collected from the infiltrated plants are germinated, and progeny carrying the transgene are selected. This transformation method produces progeny exhibiting stable inheritance of the transgene without the need for regeneration methods such as somatic embryogenesis or organogenesis.



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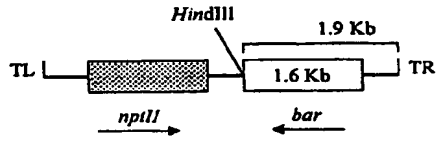

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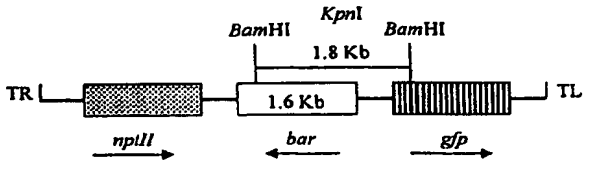

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PLANT TRANSFORMATION PROCESS

TECHNICAL FIELD OF INVENTION

This application relates to a plant transformation method and transgenic plants and plant cells originating therefrom.

BACKGROUND

Genetic transformation of higher plants promises to have a major impact on crop improvement, as well as many other areas of biotechnology. Genetic transformation can be used to produce transgenic plants carrying new genetic material stably integrated into the genome and to engineer 'designer' crops with specific traits. Various methods of genetic transformation have been developed and applied to a growing number of plant species. However, the ease and success rate of genetic transformation methods varies widely among plant species (Muller, et al. 1987. "High meiotic stability of a foreign gene introduced into tobacco by *Agrobacterium*-mediated transformation," *Mol Gen Genet* 207:171-175; Gasser, C.S. and Fraley, R.T. 1989. "Genetically engineering plants for crop improvement," *Science* 244:1293-1299; Umbeck, et al. 1989. "Inheritance and expression of genes for kanamycin and chloramphenicol resistance in transgenic cotton plants," *Crop Science* 29:196-201; Gordon-Kamm, et al. 1990. "Transformation of maize cells and regeneration of fertile transgenic plants," *Plant Cell* 2:603-618; Chabaud, et al. 1996."Transformation of barrel medic (*Medicago truncatula* Gaertn.) by *Agrobacterium tumefaciens* and regeneration via somatic embryogenesis of transgenic plants with *MtENOD12* nodulin promoter fused to the *gus* reporter gene," *Plant Cell Rep* 15:305-310; Kar, et al. 1996."Efficient transgenic plant regeneration through *Agrobacterium*-mediated transformation of chickpea (*Cicer arientinum* L.)," *Plant Cell Rep* 16:32-37; Kim, J.W. and Minamikawa, T. 1996. "Transformation and regeneration of French bean plants by the particle bombardment process," *Plant Sci* 117:131-138; Trieu, A.T. and Harrison, M.J. 1996. "Rapid transformation of *Medicago truncatula*: regeneration via shoot organogenesis," *Plant Cell Rep* 16:6-11; Bean, et al. 1997."A simple system for pea transformation," *Plant Cell Rep* 16:513-519; Cheng, et al. 1997. "Genetic transformation

of wheat mediated by *Agrobacterium tumefaciens*," *Plant Physiol* 115:971-980 ; Cheng, et al. 1997. "Expression and inheritance of foreign genes in transgenic peanut plants generated by *Agrobacterium*-mediated transformation," *Plant Cell Rep* 16:541-544; and Tingay, et al. 1997. "*Agrobacterium tumefaciens*-mediated barley transformation," *Plant J* 11:1369-1376.)

The most common and widely used method of transformation of dicotyledonous plants utilizes a bacterium, *Agrobacterium tumefaciens,* to effect gene transfer. *Agrobacterium tumefaciens* is a gram-negative, soil dwelling plant pathogen that infects its plant host and subsequently delivers and integrates part of its genetic material into the plant genome. The transferred portion of DNA is termed the T-DNA fragment, and additional genetic material can be added to the T-DNA. The additional genetic material will then be integrated into the genome along with the T-DNA. In this way, *Agrobacterium* can be used to facilitate the transfer of new genes into the plant genome (Fraley, et al. 1983. "Expression of bacterial genes in plant cells," *Proc Natl Acad Sci USA* 80:4803-4807).

While transformation with *Agrobacterium* has worked well for a number of model species such as tobacco and petunia, the approach is subject to a number of limitations. Some plant species, including many monocotyledonous plant species, are not readily susceptible to infection by *Agrobacterium* (Potrykus, I. 1990. "Gene transfer to cereals: an assessment," *Bio/Technology* 8:535-542). In these cases, alternative approaches have been used, including particle bombardment and direct gene transfer into protoplasts via electroporation, microinjection, or polyethylene glycol mediated uptake (Klein, et al. 1987. "High velocity microprojectiles for delivering nucleic acids into living cells," *Nature* 327:70-73; McCabe, et al. 1988. "Stable transformation of soy bean (*Glycine max*) by particle acceleration," *Bio/Technology* 6:923-926; Bommineni, et al. 1994. "Expression of GUS in somatic embryo cultures of black spruce after microprojectile bombardment," *J Exp Bot* 45:491-495; Christou, P. 1995. "Strategies for variety-independent genetic transformation of important cereals, legumes and woody species utilizing particle bombardment," *Eupytica* 85:13-27; Kim and Minamikawa. 1996. *Plant Sci* 117:131-138; Klein, et al. 1998. "Stable genetic transformation in intact *Nicotania* cells by the particle bombardment proeess," *Proc Natl Acad Sci USA* 85:8502-8505).

Regardless of the method of delivery of the new genetic material, it is necessary to regenerate whole fertile plants from the transformed cells. The production of stably transformed transgenic plants involves two processes: transformation of plant cells and then regeneration of those transformed cells to whole plants. In most cases, a plant tissue explant is incubated with *Agrobacterium* carrying a T-DNA containing a selectable marker gene and a 'gene of interest.' A proportion of the cells in the explant will become transformed, and whole plants are then regenerated from these cells via somatic embryogenesis or direct organogenesis. Transformants are selected by inclusion of the appropriate selective conditions in the regeneration media. The choice of tissue explant depends on the plant species. Leaf, cotyledons, hypocotyls, cotyledonary meristems, and embryos are among those that have been used successfully.

Because neither the transformation nor the regeneration are 100% effective, the chance of obtaining a transformed plant depends on these two processes occurring consecutively in the same cell. In many cases, the production of transgenic plants is prevented due to the inability to regenerate plants from those tissues susceptible to transformation. For species in which somatic embryogenesis is a viable method of regenerating plants, there are other limitations. Plants regenerated via somatic embryogenesis may show significant somatic variation, altered ploidy, phenotypic abnormalities and poor fertility (Bean, et al. 1997. *Plant Cell Rep* 16:513-519). While regeneration via direct organogenesis overcomes some of these problems, not all plants can be regenerated in this way. Finally, although transformation of many crop plants is possible, it is usually achieved in highly regenerable lines or cultivars, and the elite agriculturally important lines are not usually amenable to transformation. Therefore, introduction of a desired trait into the elite lines has been limited to subsequent traditional breeding methods following transformation of parental lines.

Often, large numbers of transgenic plants are needed. For example, in order to develop a plant line expressing a new trait, it is desirable to produce a large number of transgenic plants from which the best expressing line can be selected. Integration of the T-DNA fragment into the plant genome is a random event, and, therefore, transgenic plants will vary in the levels of expression of the introduced gene due to position effects (Ulian, et al. 1994. "Expression and inheritance pattern of two foreign genes in petunia,"

Theor Appl Genet 88:433-440). Large numbers of independent transformants are also needed for development of libraries of knock out or activation tagged lines.

The only plant which has been successfully transformed with a high degree of ease and efficiency is *Arabidopsis thaliana*, a model plant used widely for genetic and molecular analyses of plant developmental processes. A direct method of transformation has been developed for *Arabidopsis thaliana* (Bechtold, et al. 1993. "In planta *Agrobacterium* mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants," *Comptes Renus de l'Academie des Sciences Serie III Sciences de la vie* 316.). In this transformation process, the plant is (1) grown to maturity, (2) immersed in a suspension of *Agrobacterium* cells, (3) held under vacuum for a short period of time, and then (4) allowed to set seed. A proportion of the progeny is transformed. Recent data suggest that the gametophyte progenitor, gametophyte, or fertilized embryos are the targets (Bechtold, N. and Pelletier, G. 1998. "*In planta Agrobacterium*-mediated transformation of adult *Arabidopsis thaliana* plants by vacuum infiltration," *Methods Mol Biol* 82:259-266). Although Bechtold's method has been tried in other species including *Brassica napus* and *Beta vulgaris*, these attempts reportedly have been unsuccessful (Siemens, J. Scheiler, O. 1996. "Transgenic plants: genetic transformation-recent developments and the state of the art," *Plant Tissue Culture and Biotechnology* 2:66-75).

Leguminous crops such as peas, soybean, bean, alfalfa, peanut, chick pea, pigeon pea and clover have widespread economic importance throughout the world as protein sources for plants and animals, and in association with rhizobia, they are also an essential component of the global nitrogen cycle. For example, soybeans (*Glycine max*) are a major source of protein in animal and human food, and soybean oil is the most widely used edible oil in the world. The productivity, and therefore value, of a wide range of leguminous crops could be increased by the introduction of traits such as disease resistance, herbicide resistance, insect resistance, reduced levels of tannins and lignin (forage legumes), and improved protein and lipid quality. For example, the soybean cyst nematode causes losses in yield of up to one billion United States dollars per year. With the recent cloning of a beet cyst nematode resistance gene and a potato cyst nematode resistance gene (Williamson, V.M. 1999. *Curr Opin Plant Biol* 2:327-31), strategies are now being explored for genetically engineering resistance in plants.

While there have been some attempts to introduce improved traits into leguminous crops via genetic engineering, the current transformation methods involving tissue culture are exceedingly labor intensive and inefficient. In particular, the large seed grain legumes, such as pea, bean, and soybean have proved very difficult to transform, and tissues susceptible to transformation have proven difficult to regenerate. [Bingham. et al. 1975. "Breeding alfalfa which regenerates from callus tissue in culture," *Crop Science* 15:719-721; Hinchee, et al. 1988. "Production of transgenic soybean plants using *Agrobacterium*-mediated DNA transfer," *Bio/Technology* 6:915-922; Schroeder, et al. 1993. "Transformation and regeneration of two cultivars of pea (*Pisum sativum* L.)" *Plant Physiol* 101:751-757; Chabaud, et al. 1996. *Plant Cell Rep* 15:305-310; Kar, et al. 1996. *Plant Cell Rep* 16:32-37; Kim and Minamikawa. 1996. *Plant Sci* 117:131-138; Trieu and Harrison. 1996. *Plant Cell Rep* 16:6-11; Bean, et al. 1997. *Plant Cell Rep* 16:513-519; Cheng, et al. 1997. *Plant Cell Rep* 16:541-544; and Dillen, et al. 1997. "Exploiting the presence of regeneration capacity in the Phaseolus gene pool for *Agrobacterium*-meditated gene transfer to the common bean. *Mededelingen-Faculteit-Landbouwkundige-en-Toegepaste-Biologische-Wetenschappen-Universiteit-Gent* 62:1397-1402].

In an alternative approach, it was shown that cells within a soybean meristem can be transformed by particle bombardment. However, this leads to chimeric plants with transformed sectors. Some of these sectors will eventually give rise to seed, and the seed will carry the transgene (McCabe, et al. 1988. *Bio/Technology* 6:923-926; Chowrira, et al. 1995. "Electroporation-mediated gene transfer into intact nodal meristems *in planta*: generating transgenic plants without in vitro tissue culture," *Molecular Biotechnology* 3:17-23; and Chowrira, et al. 1996. "Transgenic grain legumes obtained by *in planta* electroporation-mediated gene transfer," *Molecular Biotechnology* 5:85-96). While this procedure has enabled the production of transgenic soybean, it is very labor intensive because numerous meristems need to be bombarded to have a realistic chance of obtaining any transgenic seeds.

Medicago truncatula Gaertn. (barrel medic) is a diploid, autogamous, annual medic that is grown as a pasture legume in a number of regions throughout the world, including Mediterranean areas, South Africa and Australia (Crawford, et al. 1989.

"Breeding annual *Medicago* species for semiarid conditions in Southern Australia," *Adv Agron* 42:399-437). In Australia, the annual medics are the main legume found on over 50 million hectares of agricultural land, and a variety of species and ecotypes have been developed. The first commercial cultivar of *M. truncatula* was sown in 1938, and this species has been favored due to its ability to tolerate both low rainfall and high lime soils (Crawford, et al. 1989. *Adv Agron* 42:399-437). *Medicago truncatula* also is emerging as a model legume for studies of the nitrogen-fixing Rhizobium/legume symbiosis and the arbuscular mycorrhizal symbiosis (Cook, et al. 1995. "Transient induction of a peroxidase gene in *Medicago truncatula* precedes infection by *Rhizobium meliloti*," *Plant Cell* 7:43-55; van Buuren, et al. 1998. "Novel genes induced during an arbuscular mycorrhizal (AM) symbiosis between *M. truncatula* and *G. versiforme*," *MPMI* 12:171-181). The attributes that make *M. truncatula* a useful model plant for molecular and genetic analyses include its small genome (4.5 times larger than *Arabidopsis*), rapid life cycle, and relatively small physical size (Barker, et al. 1990. "*Medicago truncatula*, a model plant for studying the molecular genetics of the *Rhizobium*-legume symbiosis," *Plant Mol Biol Rep* 8:40-49). In addition, it can be transformed via *Agrobacterium* and regenerated via somatic embryogenesis, or alternatively, by direct organogenesis (Thomas, et al. 1992. "Genetic transformation of *Medicago truncatula* using *Agrobacterium* with genetically modified Ri and disarmed Ti plasmids," *Plant Cell Rep* 11:113-117; Chabaud, et al. 1996. *Plant Cell Rep* 15:305-310; Trieu and Harrison. 1996. *Plant Cell Rep* 16:6-11; Hoffmann, et al. 1997. "A new *Medicago truncatula* line with superior in vitro regeneration, transformation, and symbiotic properties isolated through cell culture selection," *Mol Plant-Microbe Interact* 10:307-315).

Although *Agrobacterium*-mediated transformation with regeneration via somatic embryogenesis or direct organogenesis is a viable approach, these methods are very labor intensive, not very efficient, and in some cases, very slow. While these approaches may be suitable for the generation of small numbers of transgenic plants, they cannot be used to generate the large numbers of lines required for many genetic approaches and high through-put systems, such as T-DNA mutagenesis or activation tagging.

An *Agrobacterium*-mediated in planta transformation method has now been found wherein plants are exposed directly to *Agrobacterium* cells, eliminating the need for

preparation of cell or callus cultures, explants or other materials for treatment. Moreover, regeneration steps are eliminated; treated plants are allowed to mature and set seed, and many of the progeny grown from such seed are transgenic plants representing various insertional events. If desired, transgenic progeny can be selected from nontransgenic progeny by use of appropriate selection systems, such as the *bar* gene and PPT herbicide. The direct in planta transformation method of the present invention provides high efficiency, low labor input, and large numbers of transgenic plants representing independent transformation events without problems associated with preparation and transformation of isolated cells or tissues and without difficulties associated with subsequent regeneration of whole plants such as by somatic embryogenesis or direct organogenesis.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1A-1C are schematic representations of the T-DNAs used in the transformation experiments.

Fig. 1A is a schematic representation of the pSKI006 construct.

Fig. 1B is a schematic representation of the pSLJ525 construct.

Fig. 1C is a schematic representation of the pBIN*mgfp-ER-bar* construct.

SUMMARY OF THE INVENTION

In one aspect, the present invention is a method for direct plant transformation using plants and *Agrobacterium* comprising: contacting the aerial portions of at least one plant at the time of flowering with *Agrobacterium* cells which harbor a vector that enables the *Agrobacterium* cells to transfer T-DNA containing at least one gene or gene fragment to the plant; and applying a vacuum to the plant or plant portions in contact with the *Agrobacterium* cells at one point in time, the vacuum being of sufficient strength to force the *Agrobacterium* cells into intimate contact with the plant such that the *Agrobacterium* cells transfer the T-DNA to cells of the plant at a second point in time to form a transformed plant, wherein the first point in time and the second point in time are either the same or different. In a preferred method, the vector comprises a selectable

marker gene. A preferred selectable marker gene is a herbicide resistance gene. A preferred herbicide resistance gene is a *bar* gene.

In another aspect, the present invention is a method for direct transformation of a plant comprising: vernalizing and germinating initial seed to form a plant, contacting the aerial portions of the plant at the time of flowering with *Agrobacterium* cells which harbor a vector that enables the *Agrobacterium* cells to transfer T-DNA containing at least one gene or gene fragment to the plant; and applying a vacuum to the plant or plant portions in contact with the *Agrobacterium* cells at one point in time, the vacuum being of sufficient strength to force the *Agrobacterium* cells into intimate contact with the plant such that the *Agrobacterium* cells transfer the T-DNA to cells of the plant at a second point in time to form a transformed plant, wherein the first point in time and the second point in time are either the same or different. In a preferred method, the vector comprises a selectable marker gene. A preferred selectable marker gene is a herbicide resistance gene. A preferred herbicide resistance gene is a *bar* gene.

In another aspect, the present invention is a method for direct plant transformation using plants and *Agrobacterium* comprising: contacting the aerial portions of at least one plant at the time of flowering with *Agrobacterium* cells which harbor a vector that enables the *Agrobacterium* cells to transfer T-DNA containing at least one gene or gene fragment to the plant; applying a vacuum to the plant or plant portions in contact with the *Agrobacterium* cells at one point in time, the vacuum being of sufficient strength to force the *Agrobacterium* cells into intimate contact with the plant such that the *Agrobacterium* cells transfer the T-DNA to cells of the plant at a second point in time to form a transformed plant, wherein the first point in time and the second point in time are either the same or different; allowing the transformed plant to grow to maturity and set seed; germinating the seed to form progeny; and selecting for progeny expressing the transferred gene. In a preferred method, the vector comprises a selectable marker gene. A preferred selectable marker gene is a herbicide resistance gene. A preferred herbicide resistance gene is a *bar* gene.

In another aspect, the present invention is a method for direct transformation of a plant comprising: vernalizing and germinating initial seed to form a plant; contacting the

aerial portions of the plant at the time of flowering with *Agrobacterium* cells which harbor a vector that enables the *Agrobacterium* cells to transfer T-DNA containing at least one gene or gene fragment to the plant; applying a vacuum to the plant or plant portions in contact with the *Agrobacterium* cells at one point in time, the vacuum being of sufficient strength to force the *Agrobacterium* cells into intimate contact with the plant such that the *Agrobacterium* cells transfer the T-DNA to cells of the plant at a second point in time to form a transformed plant, wherein the first point in time and the second point in time are either the same or different; allowing the transformed plant to grow to maturity and set seed; germinating the seed to form progeny; and selecting for progeny expressing the transferred gene. In a preferred method, the vector comprises a selectable marker gene. A preferred selectable marker gene is a herbicide resistance gene. A preferred herbicide resistance gene is a *bar* gene.

In another aspect, the present invention is a method for direct plant transformation using plants at the time of flowering and *Agrobacterium* comprising: contacting the aerial portions of at least one plant with a mixture of *Agrobacterium* cells, the mixture comprising cells from a *Agrobacterium* strain harboring a vector with a first DNA fragment and cells from the *Agrobacterium* strain harboring the vector with a second DNA fragment, wherein the vector enables the *Agrobacterium* cells to transfer T-DNA to cells of the plant; applying a vacuum to the plant portions in contact with the mixture of *Agrobacterium* cells at a first point in time, the vacuum being of sufficient strength to force the *Agrobacterium* cells into intimate contact with the plant such that the *Agrobacterium* cells transfer T-DNA to cells of the plant at a second point in time to form a transformed plant, wherein the first point in time and the second point in time are the same or different. In a preferred method, the vector comprises a selectable marker gene. A preferred selectable marker gene is a herbicide resistance gene. A preferred herbicide resistance gene is a *bar* gene.

In another aspect, the present invention is a method for direct transformation of a plant at the time of flowering comprising: vernalizing and germinating initial seed to form the plant; contacting the aerial portions of the plant with a mixture of *Agrobacterium* cells, the mixture comprising cells from a *Agrobacterium* strain harboring a vector with a first DNA fragment and cells from the *Agrobacterium* strain harboring the

vector with a second DNA fragment, wherein the vector enables the *Agrobacterium* cells to transfer T-DNA to cells of the plant; applying a vacuum to the plant portions in contact with the mixture of *Agrobacterium* cells at a first point in time, the vacuum being of sufficient strength to force the *Agrobacterium* cells into intimate contact with the plant such that the *Agrobacterium* cells transfer T-DNA to cells of the plant at a second point in time to form a transformed plant, wherein the first point in time and the second point in time are the same or different. In a preferred method, the vector comprises a selectable marker gene. A preferred selectable marker gene is a herbicide resistance gene. A preferred herbicide resistance gene is a *bar* gene.

In another aspect, the present invention is a method for direct plant transformation using plants at the time of flowering and *Agrobacterium* comprising: contacting the aerial portions of at least one plant with a mixture of *Agrobacterium* cells, the mixture comprising cells from a *Agrobacterium* strain harboring a vector with a first DNA fragment and cells from the *Agrobacterium* strain harboring the vector with a second DNA fragment, wherein the vector enables the *Agrobacterium* cells to transfer T-DNA to cells of the plant; applying a vacuum to the plant portions in contact with the mixture of *Agrobacterium* cells at a first point in time, the vacuum being of sufficient strength to force the *Agrobacterium* cells into intimate contact with the plant such that the *Agrobacterium* cells transfer T-DNA to cells of the plant at a second point in time to form a transformed plant, wherein the first point in time and the second point in time are the same or different; allowing the transformed plant to grow to maturity and set seed; germinating the seed to form progeny; and selecting for progeny expressing the transferred gene. In a preferred method, the vector comprises a selectable marker gene. A preferred selectable marker gene is a herbicide resistance gene. A preferred herbicide resistance gene is a *bar* gene.

In another aspect, the present invention is a method for direct transformation of a plant at the time of flowering comprising: vernalizing and germinating initial seed to form the plant; contacting the aerial portions of the plant with a mixture of *Agrobacterium* cells, the mixture comprising cells from a *Agrobacterium* strain harboring a vector with a first DNA fragment and cells from the *Agrobacterium* strain harboring the vector with a second DNA fragment, wherein the vector enables the *Agrobacterium* cells

to transfer T-DNA to cells of the plant; applying a vacuum to the plant portions in contact with the mixture of *Agrobacterium* cells at a first point in time, the vacuum being of sufficient strength to force the *Agrobacterium* cells into intimate contact with the plant such that the *Agrobacterium* cells transfer T-DNA to cells of the plant at a second point in time to form a transformed plant, wherein the first point in time and the second point in time are the same or different; allowing the transformed plant to grow to maturity and set seed; germinating the seed to form progeny; and selecting for progeny expressing the transferred gene. In a preferred method, the vector comprises a selectable marker gene. A preferred selectable marker gene is a herbicide resistance gene. A preferred herbicide resistance gene is a *bar* gene.

In yet another aspect, the present invention is a method for direct plant transformation using plants at the time of flowering and *Agrobacterium* comprising: contacting aerial portions of at least one such plant at the time of flowering with *Agrobacterium* cells which harbor a vector that enables the *Agrobacterium* cells to transfer T-DNA containing at least one gene or gene fragment and a selectable marker gene to the plant; applying a vacuum to the plant portions in contact with the mixture of *Agrobacterium* cells at a first point in time, the vacuum being of sufficient strength to force the *Agrobacterium* cells into intimate contact with the plant such that the *Agrobacterium* cells transfer the T-DNA to cells of the plant at a second point in time to form a transformed plant, wherein the first point in time and the second point in time are the same or different; allowing the transformed plant to grow to maturity and set seed; germinating the seed to form progeny; exposing the progeny to an agent enabling detection of selectable marker gene expression; and selecting for progeny expressing the selectable marker gene and at least one gene, wherein expression of the selectable marker gene and at least one gene indicates gene transfer. In a preferred method, the selectable marker gene is a herbicide resistance gene. A preferred herbicide resistance gene is a *bar* gene.

In another aspect, the present invention is a method for direct transformation of a plant at the time of flowering comprising: vernalizing and germinating initial seed to form the plant; contacting aerial portions of the plant at the time of flowering with *Agrobacterium* cells which harbor a vector that enables the *Agrobacterium* cells to

transfer T-DNA containing at least one gene or gene fragment and a selectable marker gene to the plant; applying a vacuum to the plant portions in contact with the mixture of *Agrobacterium* cells at a first point in time, the vacuum being of sufficient strength to force the *Agrobacterium* cells into intimate contact with the plant such that the *Agrobacterium* cells transfer the T-DNA to cells of the plant at a second point in time to form a transformed plant, wherein the first point in time and the second point in time are the same or different; allowing the transformed plant to grow to maturity and set seed; germinating the seed to form progeny; exposing the progeny to an agent enabling detection of selectable marker gene expression; and selecting for progeny expressing the selectable marker gene and at least one gene, wherein expression of the selectable marker gene and at least one gene indicates gene transfer. In a preferred method, the selectable marker gene is a herbicide resistance gene. A preferred herbicide resistance gene is a *bar* gene.

In yet another aspect, the present invention is a plant transformed according to the above-described methods of plant transformation.

In yet another aspect, the present invention is a seed from a plant transformed according to the above-described methods of plant transformation.

In yet another aspect, the present invention is a progeny plant from a seed obtained from a plant transformed according to the above-described methods of plant transformation.

DETAILED DESCRIPTION

A plant transformation process has now been found which utilizes vacuum infiltration of plants grown from vernalized seed to flowering to introduce *Agrobacterium* T-DNA carrying a selectable marker gene and the gene(s) of interest into the plants. A plant at flowering as used herein is defined as a plant from about the beginning of first flower bud formation to about the time of last flower set. The transformation methods described herein can be applied to flowering portions of any plant, including dicots and monocots which can be successfully transformed by *Agrobacterium*-mediated gene transfer. In particular, leguminous plants are transformed at high rates of efficiency.

The transformation method described herein is generally accomplished by growing a *Agrobacterium* strain carrying a gene(s) of interest under selective conditions in liquid culture until it reaches exponential growth phase. The *Agrobacterium* cells are then pelleted by centrifugation and resuspended in a vacuum infiltration medium. The above-ground portions of plants are immersed in the *Agrobacterium* cell suspension and subjected to vacuum infiltration whereby the *Agrobacterium* cells are introduced into the plants, resulting in infiltrated plants that subsequently produce transformed seed from which a transformed plant is obtained.

The transformation of plants is accomplished through *Agrobacterium*-mediated gene transfer. *Agrobacterium* strains useful in the transformation of a plant include any aggressive strain which, upon contact with a transformable plant cell, is capable of transferring T-DNA into the cell for integration into the plant's genome. In the transformation method described herein, the *Agrobacterium* strain can carry one plasmid with multiple gene(s) of interest. Alternatively, transformation is performed using a mixture of *Agrobacterium* cells in which the vector carries different fragments of DNA, e.g., selected fragments from a specific DNA library. To achieve the optimum transformation rate in a given plant, the *Agrobacterium* strain which provides the greatest number of transformed plants is selected. For leguminous plants, *Agrobacterium tumefaciens* EHA105, ASE1, and Gv3101 strains are preferably utilized. The gene(s) of interest can be transformed into the *Agrobacterium* by any means known in the art. For example, a DNA fragment modified to contain the gene(s) of interest can be inserted into the T-DNA of an *Agrobacterium* Ti plasmid which also contains genes required to generate the transformed state.

Other modifications of the *Agrobacterium* plasmid T-DNA can be made to assist in the transformation process. For example, to distinguish progeny seedlings which are successfully transformed, a selectable marker gene can be incorporated into the T-DNA of *Agrobacterium* plasmid. Selectable marker genes useful in the transformation methods described herein include any selectable marker gene which can be incorporated into the *Agrobacterium* T-DNA and upon expression, can distinguish transformed from non-transformed progeny. Exemplary selectable markers include a neomycin transferase gene or phosphinothricin acetyl transferase (*bar*) gene. For example, a preferable selection

marker is the *bar* gene encoding phosphinothricin acetyl transferase which confers resistance to phosphinothricin-based herbicides. Preferably, the selection marker gene and gene(s) of interest are incorporated into any vector suitable for use with transforming *Agrobacterium* strains. For example, the binary vector, pBINmgfp-ER (Haseloff et al., 1997. "Removal of a cryptic intron and subcellular localization of green fluorescent protein are required to mark transgenic Arabidopsis brightly," *Proc Natl Acad Sci USA* 94:2122-2127) can be modified wherein a copy of a phosphinothricin acetyl transferase (*bar*) gene is inserted, under the control of a 35S promoter and octopine synthase 3' sequences, into the *Hin*dIII site of the T-DNA. The *bar* gene encodes phosphinothricin acetyl transferase which confers resistance to phosphinothricin-based herbicides, such as Ignite® (AgroEvo, Frankfurt, Germany). This selectable marker enables easy selection of transformed progeny plants: upon spraying the plants with phospinothricin (PPT) containing herbicides, only transformed plants containing the *bar* gene survive exposure to the herbicide.

In the plant transformation process, the starter seeds can be pretreated to optimize their germination and are vernalized to prepare the resulting plants for transformation. Surface-sterilization of the seeds is preferred to remove any interfering microorganisms which might infect the germinating seed. Any sterilization means which does not deleteriously affect the seeds can be used. Exemplary methods include use of aqueous 20-30% sodium hypochlorite or 70% ethanol. Preferably, the seeds are sterilized in a solution of 30% sodium hypochlorite and 0.1% Tween 20 for approximately 5 minutes and then thoroughly rinsed to remove the sterilizing solution. Preferably, sterile double distilled or deionized water, or water with reduced oxidizable carbon following reverse osmosis, ion exchange and/or activated charcoal treatment is used to rinse the seeds. Some seeds, for example *M. truncatula*, experience a prolonged dormancy period resulting in delayed germination. These seeds can be treated by a scarification process capable of breaking the dormancy. For example, cracking or scratching the seed coat, soaking the seed to soften the seed coat, or a controlled acid treatment can be utilized. Preferably, a treatment in concentrated sulfuric acid for approximately 10 minutes followed by thorough rinsing to remove the acid is utilized. Preferably, sterile double

distilled or deionized water, or water with reduced oxidizable carbon following reverse osmosis, ion exchange and/or activated charcoal treatment is used to rinse the seeds.

Seeds are then vernalized. Vernalization is an essential step in the transformation process of the present invention. It is expected that any vernalization method can be used. The preferred vernalization method is to place the rinsed seeds onto sterile filter paper moistened with sterile distilled water and to incubate the seeds at 4°C for 14 days in the dark, periodically adding water to the filter paper to keep it damp.

After pretreatment and vernalization, the germinating seeds are planted in a medium capable of supporting growth of plants to flowering. A preferred method is to plant seeds in 11 cm diameter pots containing Metro Mix 250 (W.R. Grace Co., Cambridge, MA) or similar mix, with about 9 plants per pot. Typically, at least four pots of vernalized plants are prepared for each construct. The plants are grown until the plants have begun to flower and have a few flowers and a few unopened flower buds, approximately 4 weeks for *M. truncatula*. The growing plants are fertilized with a complete fertilizer during growth, such as with MIRACLE-GRO® (Stern's Nurseries, Inc.). In particular, the plants must be watered well for two days prior to infiltration.

The optimum age of plants for vacuum infiltration may vary for different species. In general, plants with some flowers and some unopened flower buds are sufficiently mature. However, since plants develop at different rates, vacuum infiltration can be optimized for a specific plant by screening plants at various stages of flowering using the methods disclosed herein and determining the stage at which the transformation efficiency is maximized. The time and temperature of incubation can also be adjusted to provide optimum conditions for a specific variety. For example, approximately 4 weeks after the vernalized, germinating seeds of *M. truncatula* are planted in growth medium, plants have begun to flower and are sufficiently matured for vacuum infiltration.

A few days prior to vacuum infiltration, the transforming *Agrobacterium* is subcultured on a general plated growth medium preferably containing appropriate antibiotics to distinguish transformed *Agrobacterium* cells. For example, *Agrobacterium tumefaciens* EHA105 and ASE1 carrying the *bar* gene are preferably cultured on YEP

medium as defined in Example 1 containing rifampicin (20mg/l) and kanamycin (50 mg/l). The *Agrobacterium* cultures are grown at about 28°C for about 2-3 days.

One day prior to the vacuum infiltration, a liquid *Agrobacterium* culture is prepared by aseptically transferring an appropriate inoculum into a general growth medium suitable for growing *Agrobacterium*. TY liquid medium and YEP liquid medium containing appropriate antibiotics to select for the transformed *Agrobacterium* are preferred for *Agrobacterium* EHA105 and ASE1. The liquid cultures are grown under conditions which provide the *Agrobacterium* to reach exponential growth. Preferably, the liquid culture is incubated at about 28°C in a shaker incubator at about 250 rpm overnight. It is essential to use fresh *Agrobacterium* to achieve transformation.

To provide optimal conditions for transformation, the vacuum infiltration is preferably performed using the transforming *Agrobacterium* liquid culture in exponential growth phase (OD_{600} = 1.6). The *Agrobacterium* cells in the liquid culture are pelleted by centrifugation and resuspended in two volumes of a vacuum infiltration medium (e.g., *Agrobacterium* cells grown in 15ml liquid culture, pelleted by centrifugation, and then resuspended in 30ml vacuum infiltration medium). Any plant growth medium capable of supporting the infiltration process and the *Agrobacterium* within the plant while being compatible with plant growth can be used as the vacuum infiltration medium. More preferably, the vacuum infiltration medium comprises acetosyringone which induces the vir genes of the *Agrobacterium*. For leguminous plants, the vacuum infiltration medium defined in Examples 1 and 2 is preferably utilized.

To perform the vacuum infiltration, sufficient volume of *Agrobacterium* suspension in the vacuum infiltration medium is added to a container sufficiently large to accommodate immersion of the above ground portions of plants in the *Agrobacterium* suspension. For example, 250 ml of suspension is added to an open container having the approximate dimensions of 15 cm x 10 cm, such as the cover from a blue tip pipette box. The container is placed in a vacuum chamber, and a pot containing a plant of interest is inverted and supported over the container in such a way that the aerial portions of the plant are submerged in the *Agrobacterium* suspension.

The preferred amount of vacuum to be used in the transformation process is the minimal amount necessary to force the *Agrobacterium* into the apoplastic spaces of the plants. Approximately 28 mmHg is sufficient for transforming *M. truncatula*. The time and manner in which the vacuum is applied to the plants depends upon the plant and has to be determined empirically. For *M. truncatula*, vacuum is held for about 3 minutes and then released quickly. During the vacuum process, the *Agrobacterium* suspension bubbles profusely.

Following vacuum infiltration, the pots containing the infiltrated plants are removed from the vacuum chamber and placed on their sides in a growth chamber in such a manner as to avoid contamination of the soil mix with *Agrobacterium* suspension. The plants are then incubated at about 18°C and 95% humidity with long days of about 16 hours of light. Light quality and intensity does not appear to be critical as either cool white lights (Sylvania F48T12, 115W) or Super Spectra lights (Sylvania) can be used. Plants are held in the growth chamber for about a week and will appear sickly during this incubation period. It is important to apply water, if necessary, only to the soil mix. Water dripping from the aerial plant portions may transfer *Agrobacterium* to the soil mix, thereby greatly increasing plant mortality.

The vacuum infiltration process is then repeated on the same plants according to the procedures given above. Again, the plants are held in the growth chamber for a week, with care taken during watering to avoid transferring *Agrobacterium* to the soil mix and roots. After the second infiltration, the plants appear unhealthy with many dead leaves, and some plants may die.

Treated plants are then grown under optimum growing conditions and allowed to set seed. For *M. truncatula*, this requires about 6 to 8 weeks. During this growth period, additional flowers may form and set seed. Regardless, all seeds from treated plants are collected. Progeny plants are grown from this seed, and many of these progeny are transformed plants.

Several methods known in the art can be used to distinguish the transformed progeny exhibiting stable inheritance of the transgene. For transgenic plants wherein the gene(s) of interest results in a visible phenotypic change, the selection can be based upon

visual examination of the progeny. For plant transformations involving *Agrobacterium*-carrying plasmids containing a selectable marker gene, the appropriate selectable agent can be applied to the progeny to select the transformants. Optionally, Southern blot analysis or PCR analysis can be used to verify the presence of the transferred gene in the genome of the transformed plants.

The plant transformation processes of the present invention are further illustrated in detail in the example provided below. While this example describes the invention, it is understood that modifications to the methods to optimize transformation of a specific plant are well within the skill of one in the art, and such modifications are considered within the scope of the invention.

Example 1: Transformation of *M. truncatula* by Vacuum-infiltration of Plants at Flowering

M. truncatula plants were transformed to incorporate the *bar* gene into the plant's genome using the transformation process of the present invention.

Preliminary Methods to Prepare Plants for Transformation

Prior to transformation, a modified version of the binary vector pBINmgfp-ER (Haseloff et al., 1997. "Removal of a cryptic intron and subcellular localization of green fluorescent protein are required to mark transgenic Arabidopsis brightly," *Proc Natl Acad Sci USA* 94:2122-2127) was made by inserting a copy of a phosphinothricin acetyl transferase (*bar*) gene, under the control of a 35S promoter and octopine synthase 3' sequences, into the *Hin*dIII site of the T-DNA to create a plasmid called pBIN*mgfp-ER-bar* (Fig. 1C). The construct was confirmed by restriction analysis and PCR analysis, and then transformed into an *Agrobacterium tumefaciens* strain EHA105 (Hood, et al. 1993. "New *Agrobacterium* helper plasmids for gene transfer to plants," *Trans Res* 2:208-218). Additional constructs in *Agrobacterium* strains were also obtained as given in Table I. The following procedure presents transformation methods using for the pBIN*mgfp-ER-bar* in the EHA105 *Agrobacterium* strain. The same procedure was followed for the other constructs with the exception that the growth medium was supplemented with appropriate antibiotics necessary for maintaining the specific plasmid.

Day 1:

Medicago truncatula Gaertn 'Jemalong' (line A17) was used for all of the experiments. The *M. truncatula* seed was sterilized and germinated as follows. The seeds were soaked in conc. H_2SO_4 for approximately 10 min. The acid was removed, and the seeds were rinsed extensively in sterile cold double distilled water. This treatment was used to break dormancy in *M. truncatula*.

The seeds were then surface-sterilized by soaking the seeds in a sterilizing solution such as 30% Clorox / 0.1% Tween 20 solution for approximately 5 min with gentle agitation. The seeds were rinsed extensively with sterile cold double distilled water.

The seeds were then vernalized on a firm water-agar (for example, 0.8%) (Sigma Chemical Co., St. Louis, MO) in petri plates. The water-agar petri plates containing the seeds were wrapped with aluminum foil and kept at 4°C for about 15 days.

About Day 14:

Vernalized seeds were planted, and plants were grown in 11 cm diameter pots, in Metro-Mix 250 or 350 (W.R. Grace Co., Cambridge, MA) with 9 plants per pot. The light level was 150μmol/m^2/s with 18 hours light/25°C and 6 hours dark/22°C. The plants were fertilized with MIRACLE-GRO® (Stern's Nurseries, Inc.) when necessary.

About Day 41:

For each of the above-mentioned *Agrobacterium tumefaciens* strains, the *Agrobacterium tumefaciens* carrying the appropriate construct was subcultured for isolation onto a fresh agar plate containing YEP medium [1 liter: 10g Bacto-peptone (Difco, Detroit, MI); 10g yeast extract; 5g NaCl; and 15g Bacto-agar (Difco, Detroit, MI) at pH=6.8 without adjusting] containing rifampicin (20 mg/l) and kanamycin (50 mg/l), and the subculture was incubated at approximately 28°C for about 2-3days.

About Day 43:

One loop, or approximately 3 large colonies of the *Agrobacterium* subculture was inoculated into about 15 ml TY liquid medium [1 liter: 5g tryptone, 3g yeast extract,

0.88g $CaCl_2 \cdot 2H_2O$ at pH=7] containing rifampicin (20 mg/l) and kanamycin (50 mg/l) and incubated on a 28°C shaker at 250 rpm overnight.

About Day 44:

The *Agrobacterium* liquid culture was grown until an exponential phase (OD_{600} 1.6) was reached. The *Agrobacterium* cells were pelleted by centrifugation and resuspended in 30 ml of flower infiltration medium. Flower infiltration media is 0.5X MS salts [1X MS salt per liter: 1.65g NH_4NO_3; 0.33g $CaCl_2$; 0.18g $MgSO_4$; 1.9g KNO_3; 0.17g KH_2PO_4; 6.2mg H_3BO_3; 0.025mg $CoCl_2 \cdot 6H_2O$; 0.025mg $CuSO_4 \cdot 5H_2O$; 37.3mg Na-EDTA; 16.9mg $MnSO_4 \cdot H_2O$; 0.25mg Na_2MoO_4; 0.83mg KI; 27.8mg $FeSO_4 \cdot 7H_2O$; and 8.6mg $ZnSO_4 \cdot 7H_2O$], 1X Gamborg's vitamins [per liter: 100mg myo-inositol; 1mg nicotinic acid; 1mg pyridoxineHCl; and 10mg thiamine HCl], 0.04μM benzylamino-purine (BAP) and 0.02% Silwet77 (OSI Specialities, Inc., Danbury, CT, USA), pH 5.7.

Method of Plant Transformation for Plants at Time of Flowering

Vernalized seedlings were grown until the plants had small flower buds and a few opened flowers. This occurs approximately 4 weeks after planting. The plants were watered heavily on the day before infiltration took place. To infiltrate the plants, the pots were inverted and the above ground portion of the plant submerged in a container filled with a suspension of *Agrobacterium* in flower infiltration media. Usually the soil is held in the pot by the roots; however; if the soil appeared loose the pot was packed with cotton wool to ensure that the soil did not fall out. The pot and tray were placed in a vacuum chamber and a vacuum drawn to 25 mmHg and held for 3 minutes. The vacuum was released very rapidly and the procedure repeated once. Following two exposures to vacuum some of the plant leaves became dark and water soaked. The pots were removed from the *Agrobacterium* and placed on their sides in a tray to prevent *Agrobacterium* from entering the soil. The tray and pots were transferred to a growth chamber set at 18°C, 95% humidity, 16 hour days with 4 cool white lights (Sylvania, F48T12, 115W). The plants were incubated in the chamber for a week After two to three days the pots were placed in an upright position again. The pots were not watered during this time. After a week, the infiltration process was repeated and the plants returned to the growth chamber for a week. In some cases the plants required water during the second week and

this was applied carefully to the bottom of the pots. After the second week, the plants were returned to normal growing conditions and allowed to set seed. Transformants were selected in the subsequent generation.

About Week 6-8

The infiltrated plants were permitted to mature and seed. The seeds were then collected and germinated under conditions optimal for germination, i.e., a short cold treatment (4°C) for three-four days on a damp filter paper, left at room temperature for 1-2 days, and then planted in soil (Metro-Mix 350). When the progeny seedlings had grown to the first trifoliate stage (approximately 15 days old), they were sprayed with Ignite (AgrEvo, Wilmington, DE) diluted to contain 80 mg/L phosphinothricin (PPT) (~1/7000 dilution of 600mg/ml solution stored at -20°C). The non-transformed seedlings began to die in approximately five days. Two weeks later, the seedlings were sprayed with 560 mg/L PPT. Resistant progeny seedlings were further analyzed for the presence of the *bar* and *nptII* genes. The results are shown in Table I. These results show the efficiency of transformation ranging from 12.7% to 76.4% for the various transformation experiments.

Table I: Transformants Resulting from Transformation of *M. truncatula* via Infiltration at Flowering with *Agrobacterium*

Experiment	Construct[a] & *Agrobacterium*	No. of Plants Infiltrated	Approx. No. of Seed Collected	No. of Seedlings Germinated	No. of Seedlings Resistant to PPT	% Transformation [b]
TS1	pSLJ525/ASE1	36	ND [c]	ND	2	ND
TS2	pSKI006–Mt4 /ASE1	36	ND	ND	16	ND
TS3	pSLJ525/ASE1		ND	ND	3	ND
T86-1	pSKI006/ASE1	36	460	99	21	21.2
T86-2	pSLJ525/ASE1	36	470	182	23	12.6
T86-4	pBIN*mgfp-ER-bar*/ EHA105	36	85	17	13	76.4

[a] Selectable marker gene=*bar* gene.

[b] The majority of the transformants have been analyzed by Southern blot analysis and, in some instances, progeny analysis.

[c] ND= Not determined.

Following the first successful transformation experiments (TS1-TS3 in Table 1), in which three sets of transgenic plants were obtained, three additional experiments were undertaken (TS86-1, TS86-2 and TS86-4). Thirty-six plants were infiltrated in each experiment, and the seed from the 36 plants was collected as a single pool. Overall, the number of seeds collected was low, as was the viability; however, in these experiments (T86-1, T86-2 and T86-4) between 13 and 76% of the T1 seedlings were resistant to the selective agent, phosphinothricin (PPT) (Table 1), indicating successful transformation. Seedlings arising from a control experiment in which the plants were infiltrated with the infiltration media lacking *Agrobacterium* did not give rise to any PPT-resistant seedlings (data not shown). Transformants were not recovered from experiments in which the binary vector contained the *nptII* gene as a selectable marker (data not shown). Although kanamycin has been used successfully as a selective agent in *M. truncatula* tissue culture transformation procedures (Chabaud, et al. 1996. *Plant Cell Rep* 15:305-310), the seedlings displayed a high level of resistance to kanamycin, and transformants could not be distinguished easily.

Southern Blot Analysis

To confirm that the T-DNA was present in the genomes of the transformants, DNA was extracted from the majority of the transgenic plants from experiments T86-1, T86-2 and T86-4 and analyzed on Southern blots. DNA was isolated from the transformants and digested with appropriate enzymes as indicated in Fig. 1A-1C. For example, DNA from transformants from the T86-2 experiment was digested with *Hin*dIII. The digested DNA was separated by electrophoresis and blotted to nylon membranes. The membranes were probed with an internal DNA fragment (450bp) of the *bar* gene labeled with ^{32}P-dATP. The corresponding plasmid DNA was included on the blot as a positive control, and a sample of DNA from non-transformed *M. truncatula* plant was included as a negative control. All of the transformed plants resistant to phosphinothricin contained DNA fragments that hybridized to the *bar* probe, indicating that this gene is integrated into the genome. As expected the *bar* probe hybridized to the plasmid DNA but not to the DNA from the non-transformed *M. truncatula* plant. With regard to the efficiency of transformation, an average of 36% (Experiments T86-1, T86-2

and T86-4) of the progeny from plants subjected to flower transformation were transformed.

*Hin*dIII digested DNA from transgenic plants from Experiment T86-2 was also blotted and hybridized with an *nptII* probe (766bp) labeled with ^{32}P-dATP to obtain a left border analysis. In each case, the probe hybridized to DNA from the transformants and not to wildtype *M. truncatula* (A17) DNA. The majority of the transformants appeared to have identical hybridization patterns, indicating that there are a large number of sibling transformants. Additional left border analyses of a selection of these transformants confirmed these results, and only 3 of the 23 (13%) transformants were determined to be independent. These analyses demonstrate that in *M. truncatula*, flower infiltration transformation gives rise to both independent transformants and siblings. Based on similar analyses of transformants tested by Southern blot analysis, the percentage of independent transformants was between 13% and 23% for transformants arising from the infiltration of plants at flowering.

Analysis of T2 Progeny from Transformants

The progeny (T2) from four T1 transformants from experiments T86-1 and -2 were grown and analyzed for resistance to phosphinothricin. As shown in Table II, data was obtained which showed that the transgenes were inherited in a stable Mendelian fashion. The results show that the lines can be propagated past the T1 generation. In three cases, the progeny segregated for resistance to phosphinothricin, indicating that the original transformants were hemizygous. However, in one case, all of the progeny were resistant, suggesting that the original transformant was homozygous. Chi-square analyses of the segregation ratios of progeny from transformants T86-1.3, T86-2.3 and T86-2.11 are consistent with the presence of a single copy of the T-DNA in each transformant. Southern blot analyses are also consistent with this result and suggest that T86-2.3 and T86-2.11 are siblings.

Table II: Segregation Analysis (Phosphinothricin Resistance) of Progeny from a Selection of Transformants Prepared by Infiltration of Plants at Flowering

Progeny from transformants	Number of plants resistant to phosphinothricin	Number of plants sensitive to phosphinothricin	Chi-Square test against ratios (p)		
			3R:1S	15R:1S	63R:1S
T86-1.3	83	15	*	-	-
T86-2.3	98	32	**	-	-
T86-2.5	92	2	-	**	**
T86-2.11	106	45	**	-	-

** indicates a p value of ≥ 0.05.

* indicates a p value of ≥ 0.01.

- indicates a p value of ≤ 0.01.

In summary, the transformation process described herein is more efficient and less labor intensive than previously reported methods. In addition, somatic alterations are avoided, and direct introduction of genetic material into elite lines is made possible. Large numbers of transgenic plants can be generated very rapidly and efficiently, and the transgenes are stable and inherited by the subsequent generation. The major difficulty with regeneration of *Agrobacterium* transformed cells through tissue culture is avoided in the transformation procedures of the present invention, making it useful for legumes such as, soybean, bean and peas for which transformation or subsequent regeneration of *Agrobacterium* transformed cells is problematic.

Example 2: Preparation of *Agrobacterium tumefaciens* Constructs

Agrobacterium tumefaciens ASE1 and *Agrobacterium tumefaciens* EHA105 were utilized in the transformations. The following *Agrobacterium tumefaciens* strains and binary vectors were used in the experiments outlined in Table I: (1) *A. tumefaciens* strain ASE1 carrying the binary vector pSKI006 (schematic in Fig. 1A) (http://www.salk.edu /LABS/pbio-w/); (2) *A. tumefaciens* strain ASE1 carrying the binary vector pSLJ525 (schematic in Fig. 1B) (Jones et al. 1992. "Effective vectors for transformation, expression of heterologous genes, and assaying transposon excision in

transgenic plants," *Trans. Res.* 1: 285-297); (3) *A. tumefaciens* strain ASE1, carrying the binary vector pSKI006-Mt4, the pSKI006 vector containing the Mt4 gene under control of the 35S promoter (Burleigh, S.H. and M.J. Harrison. 1997. "A novel gene whose expression in *Medicago truncatula* is suppressed in response to colonization by vesicular-arbuscular mycorrhizal fungi and to phosphate nutrition," *Plant Mol Bio* 34:199-208); and (4) *A. tumefaciens* strain EHA105, carrying the binary vector pBIN*mgfp-ER-bar* (schematic in Fig. 1C) (Haseloff et al., 1997. "Removal of a cryptic intron and subcellular localization of green fluorescent protein are required to mark transgenic Arabidopsis brightly," *Proc Natl Acad Sci USA* 94:2122-2127). The addition of the *bar* gene to pBIN*mgfp-ER* was achieved as follows. A *Hin*dIII/*Hpa*I fragment was excised from pSLJ525. The *Hpa*I site was converted to a *Hin*dIII site by the addition of an *Hpa*I-*Hin*dIII linker, and then the *Hin*dIII fragment was then inserted into the *Hin*dIII site of pBIN*mgfp-ER* (Haseloff et al. 1997 "Removal of a cryptic intron and subcellular localization of green fluorescent protein are required to mark transgenic Arabidopsis plants brightly," *Proc Natl Acad Sci USA* 94: 2122-21271997) to create pBIN*mgfp-ER-bar*.

WE CLAIM:

1. A method for direct plant transformation using plants and *Agrobacterium* comprising:

contacting the aerial portions of at least one plant at the time of flowering with *Agrobacterium* cells, said *Agrobacterium* cells harboring a vector, said vector enabling said *Agrobacterium* cells to transfer T-DNA containing at least one gene or gene fragment to said plant; and

applying a vacuum to said plant portions in contact with said *Agrobacterium* cells at a first time, said vacuum of sufficient strength to force said *Agrobacterium* cells into intimate contact with said plant such that said *Agrobacterium* cells transfer said T-DNA to cells of said plant at a second time to form a transformed plant, wherein said first time and said second time are the same or different.

2. The method of Claim 1, further comprising:

allowing said transformed plant to grow to maturity and set seed;

germinating said seed to form progeny; and

selecting for progeny expressing said transferred gene.

3. The method of Claim 1, wherein said vector comprises a selectable marker gene.

4. The method of Claim 2, wherein said vector comprises a selectable marker gene.

5. The method of Claim 3, wherein said selectable marker gene comprises a herbicide resistance gene.

6. The method of Claim 4, wherein said selectable marker gene comprises a herbicide resistance gene.

7. The method of Claim 5, wherein said herbicide resistance gene comprises a *bar* gene.

8. The method of Claim 6, wherein said herbicide resistance gene comprises a *bar* gene.

9. A method for direct transformation of a plant comprising:

vernalizing and germinating initial seed to form said plant

contacting the aerial portions of said plant at the time of flowering with *Agrobacterium* cells, said *Agrobacterium* cells harboring a vector, said vector enabling said *Agrobacterium* cells to transfer T-DNA containing at least one gene or gene fragment to said plant; and

applying a vacuum to said plant portions in contact with said *Agrobacterium* cells at a first time, said vacuum of sufficient strength to force said *Agrobacterium* cells into intimate contact with said plant such that said *Agrobacterium* cells transfer said T-DNA to cells of said plant at a second time to form a transformed plant, wherein said first time and said second time are the same or different.

10. The method of Claim 9, further comprising:

allowing said transformed plant to grow to maturity and set seed;

germinating said seed to form progeny; and

selecting for progeny expressing said transferred gene.

11. The method of Claim 9, wherein said vector comprises a selectable marker gene.

12. The method of Claim 10, wherein said vector comprises a selectable marker gene.

13. The method of Claim 11, wherein said selectable marker gene comprises a herbicide resistance gene.

14. The method of Claim 12, wherein said selectable marker gene comprises a herbicide resistance gene.

15. The method of Claim 13, wherein said herbicide resistance gene comprises a *bar* gene.

16. The method of Claim 14, wherein said herbicide resistance gene comprises a *bar* gene.

17. A method for direct plant transformation using plants at the time of flowering and *Agrobacterium* comprising:

contacting aerial portions of at least one plant at the time of flowering with a mixture of *Agrobacterium* cells, said mixture comprising cells from a *Agrobacterium* strain harboring a vector with a DNA fragment and cells from said *Agrobacterium* strain harboring said vector with a second DNA fragment, said vector enabling said *Agrobacterium* cells to transfer said T-DNA to said plant; and

applying a vacuum to said plant portions in contact with said *Agrobacterium* cells at a first time, said vacuum of sufficient strength to force said *Agrobacterium* cells into intimate contact with said plant such that said *Agrobacterium* cells transfer T-DNA to cells of said plant at a second time to form a transformed plant, wherein said first time and said second time are the same or different.

18. The method of Claim 17, further comprising:

allowing said transformed plant to grow to maturity and set seed;

germinating said seed to form progeny; and

selecting for progeny expressing said transferred gene.

19. The method of Claim 17, wherein said vector comprises a selectable marker gene.

20. The method of Claim 18, wherein said vector comprises a selectable marker gene.

21. The method of Claim 19, wherein said selectable marker gene comprises a herbicide resistance gene.

22. The method of Claim 20, wherein said selectable marker gene comprises a herbicide resistance gene.

23. The method of Claim 21, wherein said herbicide resistance gene comprises a *bar* gene.

24. The method of Claim 22, wherein said herbicide resistance gene comprises a *bar* gene.

25. A method for direct transformation of a plant at the time of flowering comprising:

vernalizing and germinating initial seed to form said plant

contacting aerial portions of said plant at the time of flowering with a mixture of *Agrobacterium* cells, said mixture comprising cells from a *Agrobacterium* strain harboring a vector with a DNA fragment and cells from said *Agrobacterium* strain harboring said vector with a second DNA fragment, said vector enabling said *Agrobacterium* cells to transfer said T-DNA to said plant; and

applying a vacuum to said plant portions in contact with said *Agrobacterium* cells at a first time, said vacuum of sufficient strength to force said *Agrobacterium* cells into intimate contact with said plant such that said *Agrobacterium* cells transfer T-DNA to cells of said plant at a second time to form a transformed plant, wherein said first time and said second time are the same or different.

26. The method of Claim 25, further comprising:

allowing said transformed plant to grow to maturity and set seed;

germinating said seed to form progeny; and

selecting for progeny expressing said transferred gene.

27. The method of Claim 25, wherein said vector comprises a selectable marker gene.

28. The method of Claim 26, wherein said vector comprises a selectable marker gene.

29. The method of Claim 27, wherein said selectable marker gene comprises a herbicide resistance gene.

30. The method of Claim 28, wherein said selectable marker gene comprises a herbicide resistance gene.

31. The method of Claim 29, wherein said herbicide resistance gene comprises a *bar* gene.

32. The method of Claim 30, wherein said herbicide resistance gene comprises a *bar* gene.

33. A method for direct plant transformation using plants at the time of flowering and *Agrobacterium* comprising:

contacting aerial portions of at least one plant at the time of flowering with *Agrobacterium* cells, said *Agrobacterium* cells harboring a vector, said vector enabling said *Agrobacterium* cells to transfer T-DNA containing at least one gene or gene fragment and a selectable marker gene to said plant;

applying a vacuum to said plant portions in contact with said *Agrobacterium* cells at a first time, said vacuum of sufficient strength to force said *Agrobacterium* cells into intimate contact with said plant such that said *Agrobacterium* cells transfer said T-DNA to cells of said plant at a second time to form a transformed plant, wherein said first time and said second time are the same or different;

allowing said transformed plant to grow to maturity and set seed;

germinating said seed to form progeny;

exposing said progeny to an agent enabling detection of selectable marker gene expression; and

selecting for progeny expressing said selectable marker gene and at least one gene, said expression of said selectable marker gene and at least one gene indicating gene transfer.

34. The method of Claim 33, wherein said selectable marker gene comprises a herbicide resistance gene.

35. The method of Claim 34, wherein said herbicide resistance gene comprises a *bar* gene.

36. A method for direct transformation of a plant at the time of flowering comprising:

vernalizing and germinating initial seed to form said plant;

contacting aerial portions of said plant at the time of flowering with *Agrobacterium* cells, said *Agrobacterium* cells harboring a vector, said vector enabling said *Agrobacterium* cells to transfer T-DNA containing at least one gene or gene fragment and a selectable marker gene to said plant;

applying a vacuum to said plant portions in contact with said *Agrobacterium* cells at a first time, said vacuum of sufficient strength to force said *Agrobacterium* cells into

intimate contact with said plant such that said *Agrobacterium* cells transfer said T-DNA to cells of said plant at a second time to form a transformed plant, wherein said first time and said second time are the same or different;

allowing said transformed plant to grow to maturity and set seed;

germinating said seed to form progeny;

exposing said progeny to an agent enabling detection of selectable marker gene expression; and

selecting for progeny expressing said selectable marker gene and at least one gene, said expression of said selectable marker gene and at least one gene indicating gene transfer.

37. The method of Claim 36, wherein said selectable marker gene comprises a herbicide resistance gene.

38. The method of Claim 37, wherein said herbicide resistance gene comprises a *bar* gene.

39. A plant transformed according to the method of Claim 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37 or 38.

40. Seed from a plant transformed according to the method of Claim 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37 or 38.

41. A progeny plant from a seed obtained from a plant transformed according to the method of Claim 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37 or 38.

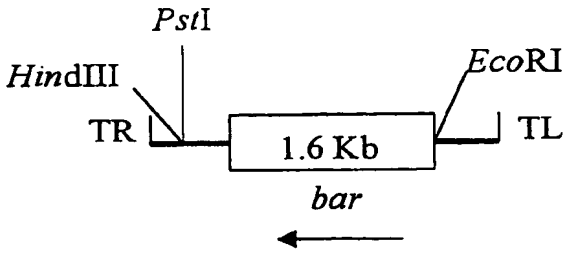

Fig 1A

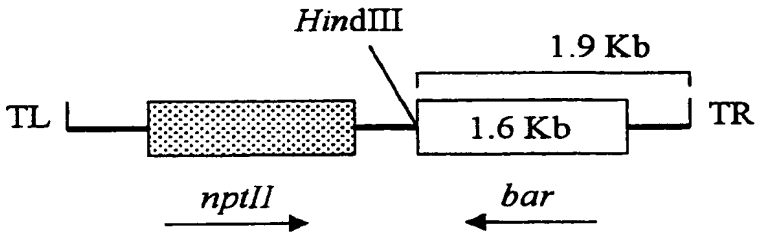

Fig 1B

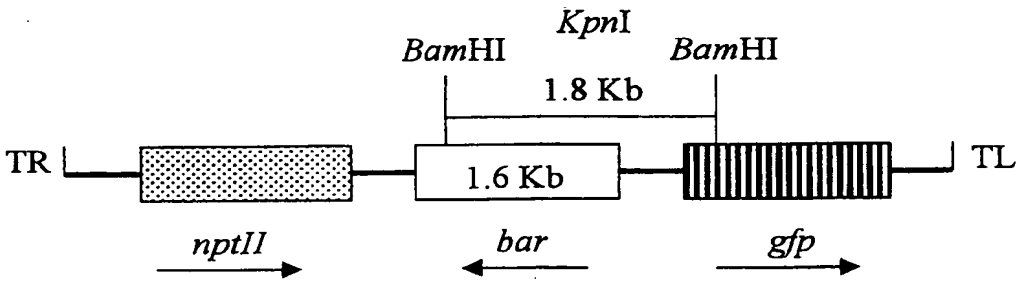

Fig 1C